Precipitation of Calcium Phosphates in the Presence of Collagen Type I on Four Different Bioactive Titanium Surfaces: an *in Vitro* Study

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ABSTRACT

Objectives: To compare the properties of calcium phosphate precipitation on four different bioactive surface preparations and one control surface in the simulated body fluid model with added collagen type I.
Material and Methods: Blasted titanium discs were treated with four different surface modifications, alkali and heat, sodium fluoride, anodic oxidation and hydroxyapatite coating. The discs were divided into five groups where one group, the blasted, served as control. The discs were immersed in simulated body fluid and collagen for 24 h, 3 days, 1 week and 2 weeks and then analysed by optical interferometry, scanning electron microscopy/energy dispersive X-ray analysis and X-ray photoelectron spectroscopy.
Results: All surfaces show small precipitates after 3 days which with longer immersion times increase. After 2 weeks the surfaces were completely covered with precipitates, and Ca/P ratios were approximately 1.3, independently on surface preparation. The fluoridated discs showed significantly ($P \leq 0.05$) higher degree of CaP after one week of immersion as compared to the other surface preparations. The collagen type I content increased with time, as reflected by increased nitrogen content.
Conclusions: The results from this study indicate that a fluoridated titanium surface may favour precipitation of calcium phosphate in the presence of collagen type I, as compared to the other surface treatments of the present study.

Keywords: biocompatible materials; collagen type I; dental implantation; titanium.

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INTRODUCTION

Since more than a decade it has been suggested that surface modified titanium implants may obtain their bone anchorage via both biomechanical and biochemical anchorage mechanisms [1,2]. The bond is in all probability predominantly biomechanical. However biochemical bonding is potentially obtained by bioactive implants. Hench [3] has defined bioactivity as "the characteristic of an implant material which allows it to form a bond with living tissues". Surface modified titanium implants may thus show bioactive properties. The surface modification can be obtained by chemical or biochemical (i.e. protein coating) treatment [4]. Alkali and heat treated titanium plates have been found to resist much higher failure loads than non-treated controls [5-7]. Moreover, some studies have presented evidence that titanium implants oxidised in a calcium hydroxide solution likewise become bioactive [8]. The wettability of the implant surface is also of importance for early osseointegration, where a more hydrophilic surface is suggested to generate a stimulating effect on healing and tissue integration to the implant surface [9]. Theoretically, the advantage with bioactive implants is that biochemical attachment is rapid and occurs at the interface, i.e. it acts at a time when proper biomechanical interlocking has not yet been developed, and initiate bone induction on the implant surface. Still, little is known about the biochemical bond at the chemical level of possible bioactive implants. The surface roughness on the implants are also of interest regarding possible ossification and it has been suggested in a systematic review on surface roughness and its effect on bone healing that a higher bone to implant contact was seen with higher surface roughness [10].

One *in vitro* model extensively used to investigate bone formation is immersion of the biomaterial in simulated body fluids (SBF), solutions with ion concentrations approximately equal to those of human blood plasma [11-16]. Depending on the nucleating capacity of the material, bone-like calcium phosphates have precipitated onto the surface. Immersion time is one factor found to influence the amount, and the crystalline degree, of the calcium phosphate precipitate [17]. Titanium surfaces modified with different preparation techniques have different surface properties, and it can be hypothesized that the different surfaces will exhibit different nucleating abilities. Alkali and heat treatment [18], anodic oxidation [19], fluoridation [20], and hydroxyapatite coatings [21] are all titanium treatments that have been suggested to produce bioactive surfaces. The actual bond has not been shown, but different indications for bioactivity have been presented. For example, when fluoridated or anodically oxidised implants have been removed from rabbit bone, the rupture has generally occurred in the bone tissue and not at the bone to implant interface [19,20]. Other researchers have evaluated bioactivity by examining nucleation and growth of apatite after immersion of implants in SBF [11]. In a review by Kokubo and Takadama [22] it was concluded that there is a correlation between apatite formation in SBF and *in vivo* bone bioactivity.

Arvidsson et al. [23] compared the formation of calcium phosphates on titanium implants with four different surface preparations by using the SBF model. A difference in the precipitation of calcium phosphates was shown between the controls and the bioactive surface types, as well as between the different bioactive surface types [23]. Recent studies have shown that four different bioactive surfaces induce faster nucleation of calcium phosphate compared to a blasted control surface in the presence of both albumin [24] and fibrinogen [25]. It was also indicated that the albumin coverage on the titanium was more rapid on the bioactive surfaces as measured in content of nitrogen [24].

Collagen is the most abundant protein in the body [26,27]. The collagens are a large family of proteins, containing at least 19 different members. They are characterized by the formation of triple helices in which three polypeptide chains are wound tightly around one another in a rope-like structure. Collagen type I is the most abundant collagen type of the human body and plays an important role in bone formation as the main organic component of bone tissue [27]. This protein is of interest because of its reported action in signal transduction and modulation in cell adhesion [28,29]. Collagen type I has also been claimed to have osteoconductive properties. Collagen on the implant surface has been reported to activate fibroblasts around the implants, favour cell adhesion to the implant surface [30], and stimulate the growth on osteoblasts adjacent to the implants [31]. In one study it was suggested that collagen coated titanium implants enhanced early bone remodelling and a tendency towards increased bone formation around the implants were reported [32].

In an *in vivo* study on nasal reconstruction using a collagen type I gel matrix it was concluded that collagen type I gel positively influenced the bone repair in large nasal defects, which showed minimal bone closure in untreated controls [33].

The aim of the present study was to compare the properties of calcium phosphate precipitation and protein adhesion on four different bioactive surface preparations and one control surface in the simulated body fluids model. The bioactive properties of the surface preparations included in the present study have earlier been described [18-21,23]. The hypothesis was that the presence of collagen type I in the simulated body fluids solution would favour precipitation on the bioactive surfaces.

MATERIAL AND METHODS

Surface preparations

In total 75 circular discs (Ø: 8 mm, thickness: 3 mm) of commercially pure titanium (grade 3) were included in the present study. The specimens were blasted with Al_2O_3 powder with a particle size of 75 µm, to increase the surface roughness. The samples were ultrasonically cleaned in diluted Extran MA01 and absolute ethanol, respectively, and dried at 60 °C for 24 hours. The discs were then divided into five groups, one group (n = 15) served as a control and was not subjected to any surface treatment prior to the SBF immersion. The other groups of samples were used for the following surface preparations.

Alkali and heat treatment

Alkali and heat treatment was performed as described in the literature [18,33,34]. The specimens (n = 15) were soaked in 5 M aqueous sodium hydroxide (NaOH) for 24 hours at 60 °C and were thereafter gently washed with distilled water before they were let to dry for 24 hours at 40 °C. The specimens were then heated in air to 600 °C by increasing the temperature by 5 °C/minute in an electrical furnace (Bitatherm, Bita Laboratory Furnaces, Israel), and were kept at 600 °C for 1 hour before being allowed to cool to room temperature in the furnace.

Anodic oxidation

Samples (n = 15) were prepared in a mixed electrolyte containing magnesium ions using the Micro Arc Oxidation (MAO) method in galvanostatic mode [35]. The electrochemical cell was composed of two platinum plates as cathodes and the titanium anode at the centre. Currents and voltages were continuously recorded at intervals of one second by an IBM computer interfaced with a DC power supply. The content of ripple was controlled to less than 0.1%. The surface properties of the oxidised group were characterised as a magnesium titanate consisting of 9 atomic % Mg, 3.4 lm of oxide thickness, 24% porosity of porous structure, and anatase plus rutile of crystal structure [36].

Fluoridation

One group of samples (n = 15) was fluoridated according to the technique of Ellingsen [20]. The samples were immersed in an aqueous solution of 0.95 M sodium fluoride (NaF) and subsequently washed twice in distilled water for 30 seconds. The samples were then allowed to dry at room temperature.

Hydroxyapatite coating

A hydroxyapatite coating was obtained by spin-coating the titanium discs (n = 15) with a stable sol which contained surfactants, water, organic solvent and crystalline nanoparticles of hydroxyapatite with a Ca/P ratio of 1.67. The diameter of the hydroxyapatite particles was approximately 10 nm. After the spin-coat procedure the discs were dried for half an hour in open air, allowing the organic solvent to evaporate. This was followed by a heat treatment at 550 ºC for 5 minutes under oxygen enriched atmosphere in order to remove all dispersing agents. The treatment resulted in a very thin hydroxyapatite coat on the titanium surface (less than 50 nm thick).

SBF immersion

The revised SBF (r-SBF) described by Oyane and co-workers [37] was used in the present study. It was prepared by dissolving 5.403 g NaCl (Merck, Darmstadt, Germany), 0.74 g $NaHCO_3$ (Merck, Darmstadt, Germany), 2.046 g Na_2CO_3 (Merck, Darmstadt, Germany), 0.225 g KCl (Merck, Darmstadt, Germany), 0.23 g $K_2HPO_4 \cdot 3H_2O$ (Merck, Darmstadt, Germany), 0.311 g $MgCl_2 \cdot 6H_2O$ (Merck, Darmstadt, Germany), 11.928 g 2-(4-(2-hydroxyethyl)-1-piperazinyl) ethanesulfonic acid (HEPES) (Research Organics Inc., Cleveland, Ohio, USA), 0.293 g $CaCl_2$ (KEBO Lab AB, Spånga, Sweden), and 0.072 g Na_2SO_4 (Merck, Darmstadt, Germany) in 1000 ml distilled water. HEPES was dissolved in 100 ml distilled water before being added to the solution, and the final pH was adjusted to 7.4 at 37 °C. Collagen solution (type I, bovine, Sigma-Aldrich Company, UK) was added result in a final concentration of 10 µg/ml.

Each specimen was immersed in 25 ml r-SBF/collagen in separate sealed polystyrene vials and kept at 37 °C.

Once every week the r-SBF/collagen was changed to freshly prepared solution. After immersion for 24 hours, 3 days, 1 week and 2 weeks, the SBF/collagen immersion was interrupted and the specimens were thoroughly rinsed with distilled water to remove any loosely attached calcium phosphate material. The specimens were then dried at room temperature and sealed in dry vials. Three of each type of surface samples (with the exception of the fluoridated control group, which consisted of two samples) was not immersed in SBF/albumin, to serve as controls for each type of surface group.

Surfaces analysis

Topographical characterization

All specimens were topographically analysed after SBF immersion with an optical interferometer (MicroXam™, PhaseShift, Tucson, Arizona, USA). The specimens of each surface type that were not immersed in SBF were also analysed, and were used as controls.

The instrument has a vertical resolution of 0.05 nm and horizontal resolution of 0.3 mm.

Each disc was measured in one area, located in the corresponding region for all discs regardless of group using a 50 times magnification objective and a zoom factor of 0.62 resulting in a measuring area of 200 x 260 μm^2.

A Gaussian filter sized 50 x 50 μm^2 was used to remove shape and waviness before surface roughness was calculated.

Thereafter the surface roughness, in terms of the following topographical parameters, was calculated:

- S_a = Arithmetic mean height deviation from a mean plane (μm).
- S_{ds} = Density of summits, i.e. the number of summits of a unit sampling area (μm^{-2}).
- S_{dr} = Developed interfacial area ratio, i.e. the ratio of the increment of the interfacial area of a surface over the sampling area (%). In other words, the increased surface area.

Mathematical descriptions of the parameters can be found in Stout et al. [38].

Calculations of group means and standard deviations for each surface preparation and time point were performed.

Scanning electron microscopy/energy dispersive X-ray analysis

For the scanning electron microscopy (SEM) analyses, a LEO Ultra 55 FEG SEM equipped with an Oxford Inca EDX system, operating at 7 kV, was used. The samples were examined without surface sputtering. Micrographs were recorded at different magnifications to investigate both the surface coverage and the morphologies of the crystals. The atomic composition was monitored using energy dispersive X-ray analysis (EDX) analysis at two different magnifications. Analyses at a low magnification were performed on a major part of the sample to describe a mean value of the atomic composition. Two samples were analysed from each surface preparation and immersion time and the mean value was calculated.

X-ray photoelectron spectroscopy

For the X-ray photoelectron spectroscopy (XPS) analyses a PHI 5000C Perkin Elmer instrument equipped with a monochromatic Al Kα X-ray source operating at 200 W was used. Each sample was analysed at two separate positions and two samples were analysed from each surface preparation and immersion time and the mean value was calculated.

Statistical analysis

Calculations of group means and standard deviations for each surface preparation and time point was performed.

The parametric comparative analysis of differences of the group means was performed with STATA (13 edition) and ANOVA, Bartlett's test for equal variance test. Statistical significance was defined at $P \leq 0.05$.

RESULTS

Surface analysis

Topographical characterization

All surface roughness results are displayed in Table 1.

S_a mean values: The control surface (B, blasted only) had higher mean values compared to the other surface preparations. The oxidised surface had the lowest mean values. The mean value of all surface preparations increased drastically after 2 weeks of immersion.

S_{ds} mean values: Higher mean values were demonstrated for all surface preparations compared to the control surface. All surfaces showed highest mean values after two weeks.

S_{dr} mean values: Similar mean values were presented after 24 h and 3 days. After 1 week the fluoridated surface had a mean value more than 50% higher compared to the other surfaces.

Table 1. Topographical results as measured with an optical interferometry

Immersion time	Surface	S_a (µm)	S_{ds} (x10^3 mm^{-2})	S_{dr} (%)
Control	B	1.09 (0.06)	142 (3)	56.6 (4.8)
	Ox	0.55 (0.36)	212 (15)	34.1 (23.6)
	F	0.98 (0.05)	176 (8)	50.8 (3.1)
	HA	1.03 (0.08)	171 (13)	58.2 (4.9)
	AH	0.97 (0.08)	243 (12)	72.8 (9.1)
24 hours	B	1.07 (0.14)	142 (3)	58.4 (20.1)
	Ox	1.03 (0.05)	191 (4)	70.3 (5.4)
	F	1 (0.03)	181 (4)	52.3 (3)
	HA	1.03 (0.07)	161 (3)	53.3 (3.6)
	AH	0.98 (0.04)	244 (7)	70.2 (4.1)
3 days	B	1.41 (0.61)	181 (11)	93 (53.4)
	Ox	1.17 (0.46)	211 (12)	99.8 (55.2)
	F	1.17 (0.46)	200 (24)	57.5 (3.4)
	HA	1 (0.06)	160 (5)	51.9 (4.4)
	AH	1 (0.03)	250 (8)	80 (3.8)
1 week	B	1.86 (1.13)	174 (11)	104.6 (67.9)
	Ox	0.97 (0.56)	230 (18)	93.1 (72.1)
	F	1.88 (0.88)	216 (7)	171.4 (126.2)
	HA	1.08 (0.06)	172 (9)	70.8 (11.2)
	AH	1.03 (0.05)	233 (4)	75.2 (5.8)
2 weeks	B	6.78 (1.52)	229 (78)	992 (340.3)
	Ox	4.79 (1.25)	284 (12)	766.6 (179.2)
	F	6.58 (0.94)	272 (9)	762.2 (169.5)
	HA	5.55 (1.3)	256 (9)	806.1 (215)
	AH	6.56 (1.34)	272 (9)	1002.3 (323.9)

The table presents mean values. Standard deviations are presented within parenthesis. Control - no immersion.
B = blasted (the control surface); Ox = anodically oxidised; F = fluoridated; HA = hydroxyapatite coated; AH = alkali and heat treated.

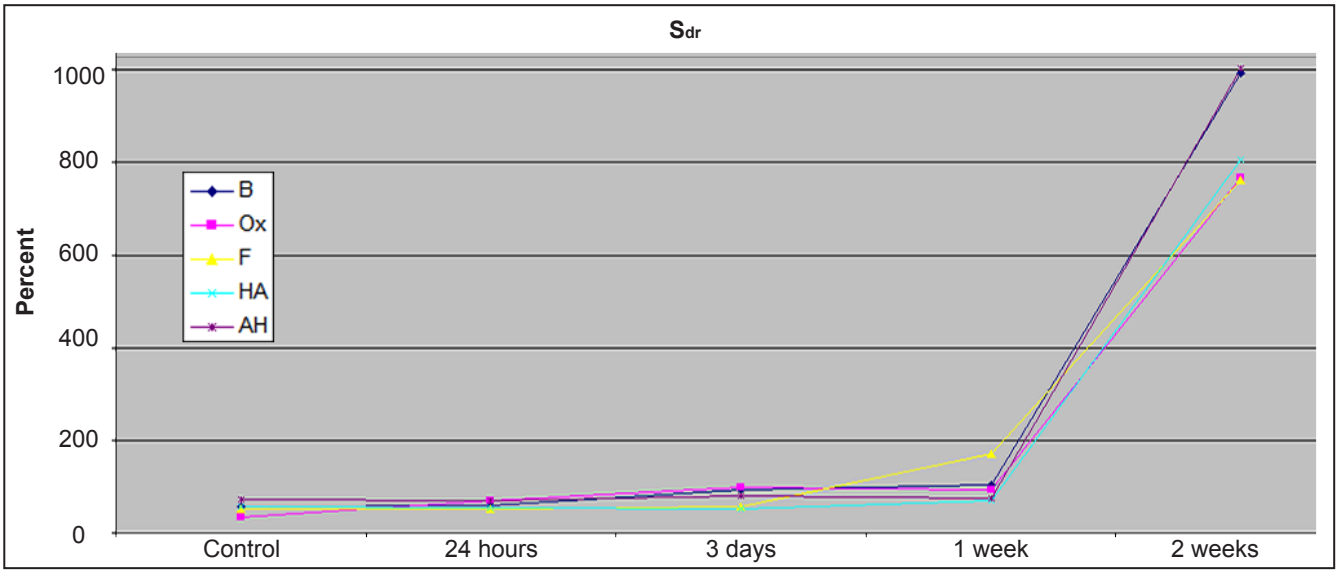

Figure 1. Mean values of S_{dr} after various immersion times.
B = blasted; Ox = anodically oxidised; F = fluoridated; HA = hydroxyapatite coated; AH = alkali and heat treated.

However, after two weeks the fluoridated surface showed the lowest mean value. All mean values increased with more than 600% after two weeks of immersion, but only the alkali and heat treated surface (AH) had a higher mean value than the control (Figure 1).

SEM/EDX

SEM

SEM images were recorded at several magnifications, ranging from 70 to 20000.

As can be seen in Figure 2, the different surfaces used in this study exhibit large differences in surface morphology. However, from the SEM images no conclusion can be drawn concerning nucleating capacity. All surfaces show small precipitates after 3 days which with longer immersion times increase. After two weeks all surfaces are fully covered as shown in Figure 3.

EDX

The element analysis indicated high levels of titanium for all surfaces at baseline. With increasing time of immersion, a decreased amount of titanium and increased coverage of phosphate, calcium and carbon was observed. The calcium content was higher on the fluoridated as well as alkali and heat treated

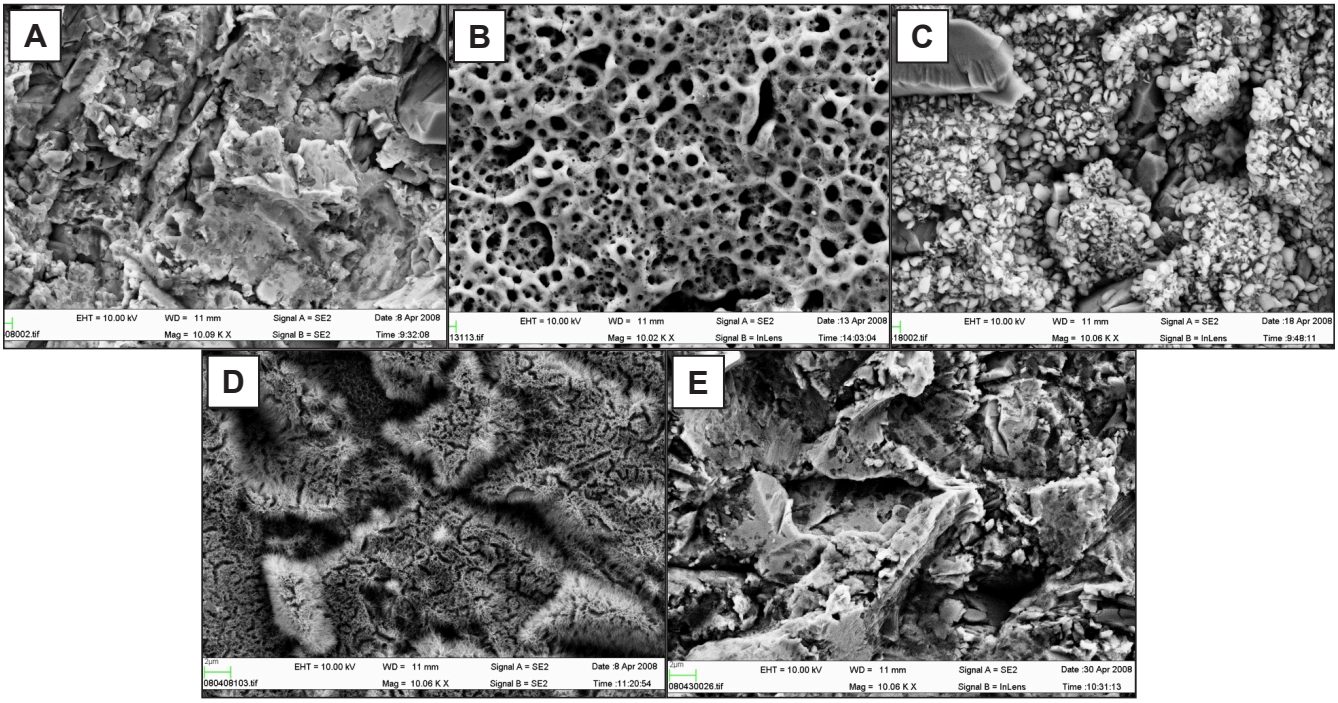

Figure 2. SEM images of different titanium surfaces. Bar = 10 µm.
A = blasted; B = anodically oxidised; C = fluoridated; D = hydroxyapatite coated; E = alkali and heat treated.

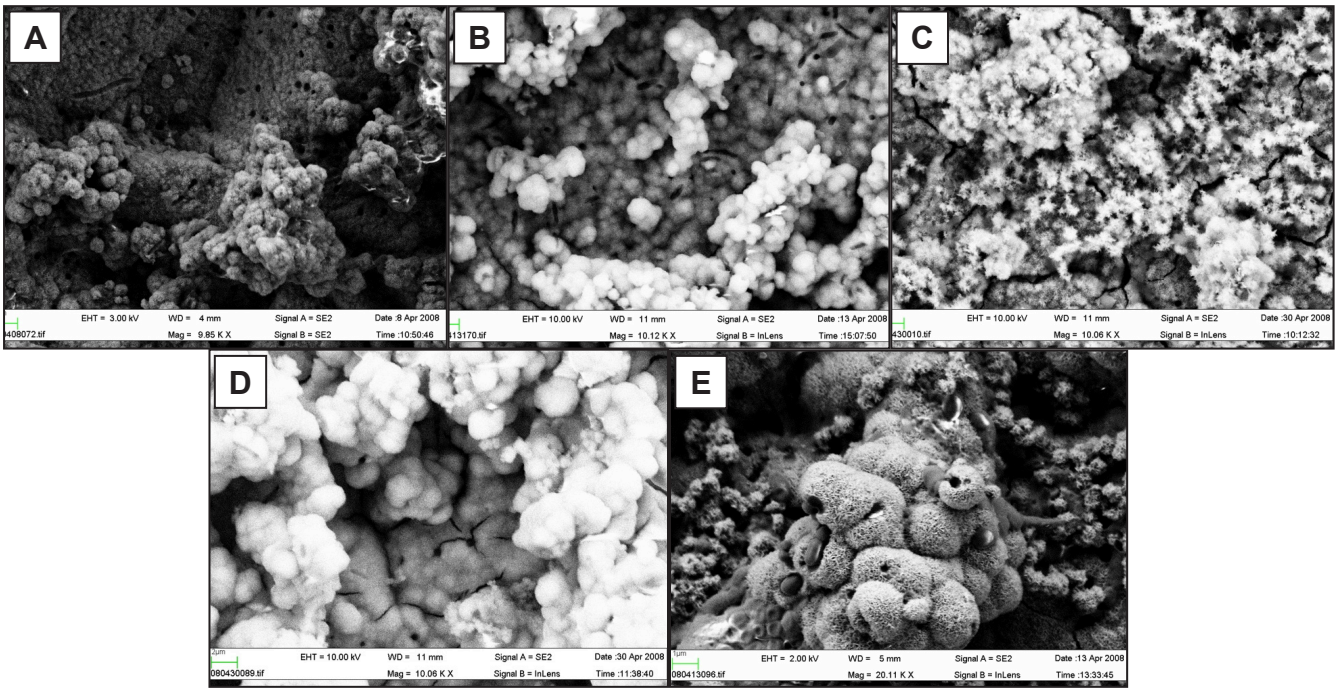

Figure 3. SEM images of different titanium surfaces after 2 weeks. Bar = 10 µm.
A = blasted; B = anodically oxidised; C = fluoridated; D = hydroxylapatite coated; E = alkali and heat treated.

samples at all time points, as compared to the other surface preparations. For the fluoridated samples, the difference was statistically significant ($P \leq 0.05$) after 1 week (Figure 4).

XPS

The analysis indicated increased amounts of calcium with increasing time however, the differences were insignificant (Figure 5). The mean Ca/P ratio was significantly higher for the fluoridated samples (Figure 6) after 24 h and 3 days compared to the mean value of the other surface treatments ($P < 0.05$). After 1 and 2 weeks the Ca/P ratios varied between 1 and 1.5 (Figure 6). The titanium coverage decreased on all samples with time (Figure 7). The nitrogen levels increased with time and were inversely related to the Ca/P ratio (Figure 8).

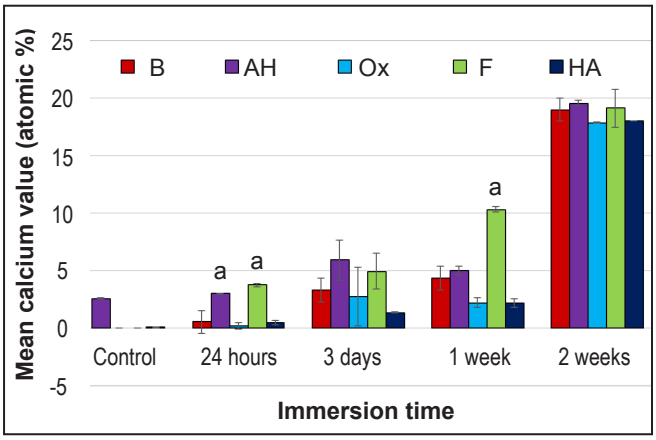

Figure 4. Mean values of calcium content on the different surfaces measured by EDX analysis.
B = blasted; AH = alkali and heat treated; Ox = anodically oxidised; F = fluoridated; HA = hydroxyapatite coated.
[a]Statistically significant at level $P \leq 0.05$, Bartlett's test.

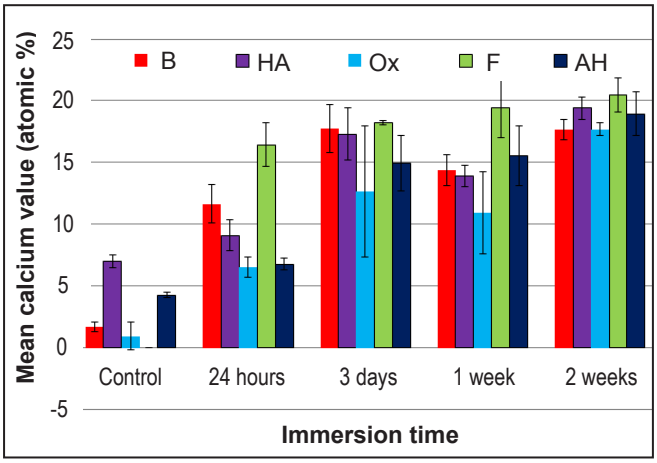

Figure 5. Mean values of calcium content on the different surfaces measured by XPS analysis.
B = blasted; HA = hydroxyapatite coated; Ox = anodically oxidised; F = fluoridated; AH = alkali and heat treated.

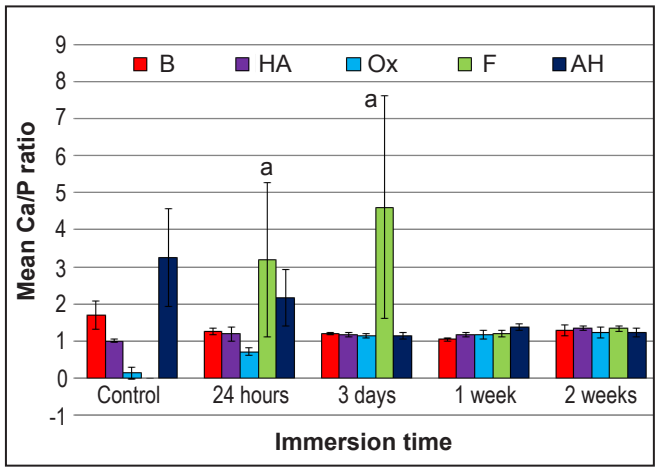

Figure 6. Mean values of Ca/P ratio on the fluoridated discs measured by XPS analysis.
B = blasted; HA = hydroxyapatite coated; Ox = anodically oxidised; F = fluoridated; AH = alkali and heat treated.
[a]Statistically significant at level $P \leq 0.05$, Bartlett's test.

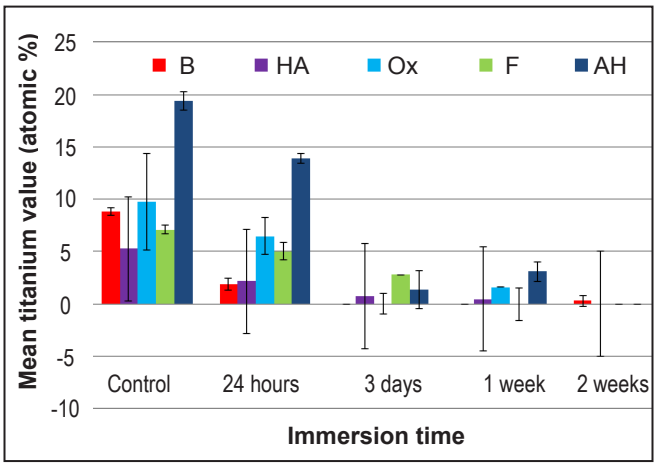

Figure 7. Mean values of titanium content on the different surfaces measured by XPS analysis.
B = blasted; HA = hydroxyapatite coated; Ox = anodically oxidised; F = fluoridated; AH = alkali and heat treated.

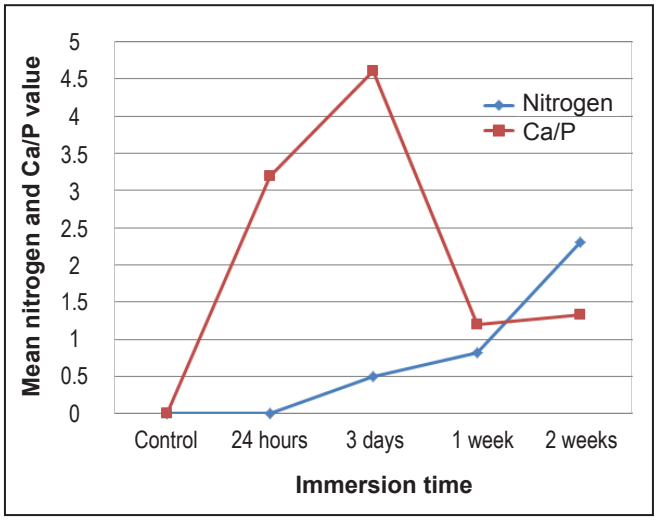

Figure 8. Mean values of nitrogen and Ca/P on the fluoridated discs measured by XPS analysis.

DISCUSSION

In the present study the formation of calcium phosphates on titanium implants with various surface preparations claimed to be bioactive, was investigated with the same settings in an SBF model with the addition of collagen type I solution.

Previously, the precipitation of calcium phosphates on surfaces has been studied *in vitro* using a variety of solutions, including SBF with varying compositions. In the present study a revised SBF [37] was chosen with an electrolyte concentration very similar to that of human plasma [39]. Regardless of the buffer used, it should be stressed that it is a simplified model for the conditions *in vivo*. However, a simplified model can facilitate the understanding of the initial phase of bone formation around titanium implants.

Furthermore, during bone formation *in vivo* there is a circulation of blood. Studies have been performed comparing results from dynamic and static SBF models, and it was found that less calcium phosphate material was formed with the dynamic model than the static model [40]. Lu and co-workers [40] also found that the surface topography influence was larger in the dynamic model than in the static model. In the present study a static SBF model was used. However, the buffer was changed after one week to avoid ion depletion and all specimens were treated according to the same protocol.

Wound healing around titanium implants is initiated by bleeding from the wound. As blood comes in contact with an implant surface it absorbs ions and proteins [41]. After a few days a fluid phase is built up around the implant, as shown by *in vivo* bone remodelling studies. It has been reported that the initial fluid phase decreases with time and is replaced by tissue [42-44]. An *in vivo* model in rabbit bone [45,46] demonstrated that after four to six weeks bone growth occurs along the upper and lower parts of the implant surface. The immersion times used in the present study were selected to cover the initial phase in wound healing around the implant and additionally to follow the precipitation events.

Results from the topographical analysis, using the optical interferometer, showed an increase of the surface area (S_{dr}) and vertical height of the irregularities (S_a) after two weeks of immersion for all surface preparations. The control surface and the alkali heat treated surface demonstrated the highest mean height values (S_a).

The precipitation of calcium phosphate on the different surface preparations was demonstrated by means of the SEM/EDX and XPS analysis. Results from the SEM/EDX analysis showed a higher Ca/P ratio on the fluoridated discs than on the other surfaces (Figure 4). After one week of immersion there was a significantly higher degree of calcium precipitate on the fluoridated specimens (Figure 5) as compared to the other surfaces. Alkali and heat treated samples showed a higher Ca/P ratio at baseline (Figure 6), but showed a lower mean Ca/P ration compared to the other surfaces with prolonged immersion time.

The XPS analysis of the fluoridated discs indicated an inverted correlation between nitrogen content and Ca/P ratio (Figure 8), where the increasing nitrogen content has been interpreted as an increased amount of proteins on the surface [47].

The titanium signal measured by XPS analysis (Figure 7) decreased on all surfaces with immersion time and after 2 weeks all titanium surfaces were completely coated and showed small amounts of detectable titanium. In earlier studies there was a higher titanium signal from titanium surfaces after two weeks in SBF without proteins [23], or with albumin [24] or fibrinogen [25], which indicates that collagen promotes precipitation of calcium phosphates. In all studies the same surface preparations were included.

The collection of data during a period of two weeks was not sufficient to determine whether the crystal growth had reached a limit i.e. a Ca/P ratio value of approximately 1.67. Thus, an extension of the immersion time with an addition of at least four weeks might have given more information about the crystallinity over time. However, compared to previous SBF studies [23-25], the Ca/P ratios after 1 and 2 weeks are relatively low in the present study (Figure 6), indicating a low degree of crystallinity. For example, after 1 week immersion in SBF without proteins, the Ca/P ratio of blasted titanium surfaces was reported to be approximately 1.7 [23]. In the present study, corresponding Ca/P ratio was measured to approximately 1 after immersion in SBF with addition of collagen type I (Figure 6).

Within the limitations of this simplified model, it is possible to study the influence of different proteins on calcium phosphates formation. Enhanced understanding of the interactions between different surface preparations and bone formation mechanisms can contribute to the further development of surface preparations that may improve the clinical outcome of bone anchored implants.

CONCLUSIONS

In this study we have described a method to coat various titanium surfaces with Ca/P and collagen type I. The results indicated that a fluoridated titanium surface may favour precipitation of Ca/P in the presence of collagen type I as compared to the other surface treatments of the present study.

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